Improved Microbial Preparations

Technical Field

The present invention relates generally to microbial preparations containing microbes having increased growth/yield potential, or increased survival/recovery rates in use. The microbial preparations are particularly suitable for inclusion in prebiotic and probiotic preparations and products including food, feed, nutraceutical and pharmaceutical products containing probiotic microorganisms, and products fermented by added microorganisms.

Background Art

Probiotic products, for example solutions, powders, tablets and capsules are orally administered for improving health. Similarly foods are consumed not only for sustenance but also for added health benefits such as through the addition of probiotic microorganisms. Animal feeds are also being prepared with added probiotic microorganisms in order to assist the growth and performance of animals. Recent trends of consuming probiotic compositions for health benefits has led to the use of probiotic microorganisms in a variety of preparations, as well as inclusion into a wide range of processed food and feed products, including processed milk-based products. Microorganisms are also added as starter cultures in order to produce a range of fermented foods e.g. milk, meat, vegetable products.

As used in this specification, a probiotic or probiotic microorganism is a live microbial feed supplement which beneficially effects the host animal by improving its intestinal microbial balance. This is the definition provided by R. Fuller (AFRC Institute of Food Research, Reading Laboratory, UK) in - Journal of Applied Bacteriology, 1989. 66, pp. 365-378 "Probiotics in Man and Animals - A Review", and has subsequently been extended to include supplements and food for humans. A probiotic or probiotic microorganism also includes a live microbial supplement or pharmaceutical preparation which can be delivered to the nasal cavity or vaginal tract which beneficially affects the host animal by improving its microbial balance at the respective site of delivery.

The constitution and quantity of the gut microflora can be influenced by conditions or stress induced by disease, life style, travel, and other factors. If microorganisms which positively effect the health and well being of the individual can be encouraged to populate the large bowel, this should improve the physiological well being of the host.

The introduction of beneficial microorganisms, or probiotics, is normally accomplished by the ingestion of the microorganisms in foods, drinks, fermented dairy products such as yoghurts, capsules, confectionary and other forms in such a way that the organism arrives in a viable condition in the large bowel or other site of interest in the host.

One problem with the inclusion of probiotic microorganisms into processed food products is that the microorganisms often cannot survive in the food product for any length of time. During production and storage of the food products, there is often a substantial decrease in the numbers of viable microorganisms. For example, usual shelf-life of milk-based products is calculated on the period of time before spoilage of the product. When probiotic microorganisms are added to these products, the shelf-life stated for the product may not be applicable with regard to delivering the desired number of microorganisms to the gut to obtain the required beneficial effect.

Other uses of microbes include biocontrol and bioremediation. Microbial preparations including microbes suitable for these uses having increased growth/yield potential, or increased survival/recovery rates would be an advantage. For instance, certain bifidobacteria and acidophilus strains are active against *Escherichia coli* and *Salmonella spp* means that the bacteria have applications as biocontrol agents. Other microbes also have biocontrol applications - for instance, various fungi and *Bacillus* species. Furthermore, Pseudomonads are known to be efficacious for bioremediation and the present invention is applicable for promoting their survival. Another commonly used bioremediation microbe is Alcaligenes.

The present inventors have made the surprising discovery that the inclusion of starch, maltose or resistant starch, in particular resistant starch in the form of, or derived from, starches containing dietary fibre, in the growth medium for the microorganisms can increase growth and yield of the microorganisms, as well as increase the survival of microorganisms in microbial preparations or starter cultures and in food and feed products during production and over the shelf-life of these products, and improve rate of survival of the microbes during transit through the digestive tract.

Dietary fibre is defined as measured by the AOAC, International (Association of the Official Analytical Chemistry) Total dietary fiber in foods: enzyme-gravimetric method (Method 985.29). Assoc. Off. Anal. Chemists, Official Methods of Analysis, 16th Ed. Arlington VA, USA, 1995. Further

Received 08 November 2000

addition of the resistant starch to the microbial preparations after growth of the microbe also further enhances robustness of the microbes, thus leading to enhanced survival of the microorganisms. The discovery is also applicable to biocontrol and bioremediation preparations as well as food fermented by the addition of microorganisms, starter cultures, since more robust starter cultures can also be produced by growth on resistant starch medium and/or addition of resistant starch to subsequent products as described above.

Disclosure of the Invention

In a first general aspect, the present invention provides an improved microbial preparation including microbes having an increased survival/recovery rate in a product or fermentation. Preferably, the product is a food, feed, nutraceutical, pharmaceutical, biocontrol or bioremediation product. Preferably, the microbial preparation comprises microbes previously grown in media based on, or containing, resistant starch such that the harvested microbes have an increased survival/recovery rate in subsequent use. One form of resistant starch particularly suitable for the present invention is starch containing resistant starch, particularly high amylose maize starches or materials derived from high amylose maize starches. In subsequent use, the preparation may be mixed with additional resistant starch to further enhance the growth/yield potential, or increased survival/recovery rate of the microbes in the product. It has been found that growth in media based on, or containing, soluble starch, maltose or resistant starch seems to enhance the microbes ability to bind to additional resistant starch added to the product therefore enhancing the growth/yield potential, or increased survival/recovery rate of the microbes.

The microbes in the improved microbial preparation according to the first aspect of the present invention are particularly unaffected by stresses including aeration, sheer, freeze drying, freezing, drying including high, medium and low water activity, elevated temperatures, low temperatures, pressure and pressure fluctuations, low pH, high pH, bile acids, moisture, high or low osmolarity, high salt, or combinations thereof. The microbial preparation according to the present invention is particularly suitable for use in probiotics, starter cultures, biocontrol or bioremediation agents.

In a second aspect, the present invention provides a process of preparing a microbial preparation having an increased survival/recovery rate in a product, the process comprising growing or culturing microbes in media

based on or containing resistant starch such that the cultured microbes will have an increased survival/recovery rate when used subsequently in a product, and harvesting the cultured microbes having an increased survival/recovery rate, wherein in subsequent use in a product, the microbes have an increased survival/recovery rate in the product compared with the same microbes grown or cultured in media without resistant starch and used in a similar product.

In a third aspect, the present invention provides an improved microbial preparation having an increased survival/recovery rate in a product prepared by the process according to the second aspect of the present invention.

In a fourth aspect, the present invention provides a product containing microbes having an increased survival/recovery rate, the product including a microbial preparation according to the first or third aspects of the present invention.

Preferably, the product is a food, feed, nutraceutical, pharmaceutical, biocontrol or bioremediation product. In one preferred embodiment, the food product also includes resistant starch so as to further enhance growth/yield potential, or survival/recovery rate of the microbes.

In a fifth aspect, the present invention relates to the use of resistant starch in microbial culture media to produce microbes having an increased survival/recovery rate in a product compared with the same microbes grown or cultured in media without resistant starch.

One form of resistant starch particularly suitable for the present invention is starch containing resistant starch. Preferably, the starches have an amylose content of at least 40% (w/w). In a preferred form the starch is from maize having an amylose content of at least 70% (w/w), at least 80% (w/w) or at least 90% (w/w). The starch can also be chemically, physically, or enzymically treated or modified. Chemical modification can be by oxidation, cross-bonding, etherification, esterification, acidification, dextrinisation, or mixtures thereof. Starches can also be treated to enhance the resistant starch content by a number of physical or chemical means. One preferred means is to heat-treat starch in the presence of moisture (heat-moisture treatment) which can be achieved by a number of processes including heating under negative, atmospheric or positive pressure under elevated moisture, or cycling techniques through different temperatures and pressures. Heating can be in the order of 100 to 180°C, preferably around 120

to 150°C and moisture levels of 10 to 80%, preferably 20 to 60%. Repeated autoclaving and rapid cooling can also be used to increase the resistant starch content of starches. It will be appreciated that these processes and conditions can be changed to achieve the desired increase in the level of resistant starch in the starch being treated. Treatment can also be by solvent extraction to remove fats and/or minerals from the starch.

There are a variety of probiotic microorganisms which are suitable for use in this invention including yeasts such as *Saccharomyces*, and bacteria such as the genera *Bifidobacterium*, *Bacteroides*, *Clostridium*, *Fusobacterium*, *Propionibacterium*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Staphylococcus*, *Leuconostoc*, *Peptostreptococcus* and *Lactobacillus*. The invention is not, however, limited to these particular microorganisms. Preferably, the starter cultures include, but not limited, to lactic acid bacteria including lactobacillus, lactococcus and streptococcus, leuconostoc, and yeasts. Preferably, the microorganisms for use in biocontrol or bioremediation products include bifidobacteria, acidophilus, fungi, *Bacillus* species, pseudomonads and Alcaligenes. It will be appreciated, however, that other species of microorganisms would also be suitable candidates for use according to the present invention.

The invention also includes microorganisms of different strains or species, including non-starch utilisers, to interact and demonstrate improved growth and/or activity in the large bowel, nasal tract or vaginal tract.

In a preferred embodiment of the first, second, third and fourth aspects of the present invention, the microbial preparations are starter cultures or probiotic preparations which can be liquid, frozen or dried. The preparations may also include food and feed products containing other microbial additives. These products include fluid-based or solid-based products. Fluid-based food products include milk-based products where the edible ingredient is one or more milk-based ingredients including whole milk, milk solids, milk fat, cream, non-fat dried milk, any other component or derivative from milk that can be used in milk-based products, water-based fluids, cereal and plant extracts such as soy-based beverages and additives. Solid-based food products include snack bars, breakfast cereals, bread, confectionary, extruded food products, muesli bars, buns, biscuits, feed pellets, coated food products, tablets, food additives, health supplements, and pharmaceutical preparations.

The food products according to the fourth aspect of the present invention include any food product that is suitable to contain and deliver probiotic microorganisms. Examples include, but not limited to, food stuffs, fruit beverages, water ices, confectionary, coatings or covertures, yoghurts, yoghurt drinks, unfermented drinks, flavoured milk drinks, modified milk drinks, ice-creams, and dairy desserts.

Standard methods employed by the art can be used to prepare the food, feed, nutraceutical or pharmaceutical products according to the fourth aspect of the present invention. The resistant starch may be added separately, in combination with one or more of the ingredients that form part of the food product. The resistant starch when added separately may interact positively and/or synergistically with other ingredients in the food, feed, nutraceutical or pharmaceutical products.

The increase in survival rate of the microbes in the product relates to an increase over the expected survival rate of the same microbe in a similar product that does not contain the resistant starch-grown microbes.

In a preferred form, the resistant starch is the Hi-maize™ and Culture Pro™ range of resistant starch products. The resistant starch can be used in growth media at a concentration of about 0.01 to 10% (w/w) and in subsequent additions during preparation of the microbial preparations and in liquid food, feed, nutraceutical or pharmaceutical products. Preferably the resistant starch is used at 0.1 to 5% (w/w) and more preferably at about 1% (w/w). The starch containing resistant starch and/or dietary fibre can be used in dry food, feed, nutraceutical or pharmaceutical products and microbial preparations at a concentration of about 0.1 to 90% (w/w) total product or preparation. Preferably, the starch is used at about 1 to 10% (w/w).

Resistant starch has been found to be particularly suitable in fluid-based foods at a concentration of 0.1 to 5% w/v, in solid-based foods at 0.1 to 15% (w/w), and in feed, nutraceutical or pharmaceutical products at 0.1 to 95% (w/w).

A further advantage of the use of resistant starch is that additional resistant starch can also be added at any stage during the processing of the food, feed, nutraceutical or pharmaceutical product. The properties of the resistant starch are not adversely effected by the processes involved in producing processed products. One distinct advantage is that there is no need to add the resistant starch in sterile form at the end of the process. The

product can undergo pasteurisation or the like without the concern of adversely effecting the starches' properties.

As used in this specification, "resistant starch" includes those forms defined as RS1, RS2, RS3 and RS4 as defined in Brown, McNaught and Moloney (1995) Food Australia **47**: 272-275. Either modified or unmodified resistant starches or mixtures thereof can be used in the present invention.

In WO 94/03049 and WO 94/14342, high amylose starches are disclosed which are resistant starches and include maize starch having an amylose content of 50% (w/w) or more, particularly 80% (w/w) or more, rice starch having an amylose content of 27% (w/w) or more, or a wheat starch having 35% (w/w) or more. Furthermore, particular granular size ranges of starches having an amylose content of 50% or more and enhanced resistant starch content, these starches including maize, barley, and legumes. This invention is not, however, limited to these forms of resistant starch. For example, other forms of resistant starch are derived from sources such as bananas and tubers such as potatoes and modified forms thereof.

Chemical modifications, such as oxidation, cross-bonding, etherification, esterification, acidification, dextrinisation and the like are well known in this art as being suitable chemical treatments. Similarly, other modifications can be induced physically, enzymically or by other means well known to those skilled in the art.

It may also be useful to modify the degree of enzyme susceptibility of the resistant starch by altering the conformation or structure of the starch. Examples include acid or enzyme thinning and cross bonding using di-functional reagents, heat/moisture treatment and thermal annealing. Modification of the starch may also be carried out by manipulation of the crystalline nature of the starch. Such modification methods are known to the art and starches produced by these methods would be suitable for the present invention.

Preferably the resistant starches are derived or obtained from corn (maize). It will be appreciated, however, that other sources of resistant starch could be used in the present invention. Examples include cereals like sorghum, wheat, barley, oats and rice, tubers like potatoes and tapioca, legumes such as peas, and others including starches derived from genetically modified plant species.

As used herein, Hi-maize™ and Culture Pro™ refers to products obtained from high amylose starch containing over 70% amylose obtained from Starch Australasia Limited. High amylose starches containing resistant starch suitable for the present invention are described in AU 660560.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples and drawings.

Brief Description of Drawings

Figure 1 shows growth of *Bifidobacterium* strain D with and without starch.

Figure 2 shows growth of *Bifidobacterium* strain E with and without starch.

Figure 3 shows growth of *Bifidobacterium* strain C with and without starch.

Figure 4 shows the survival/recovery of starch-grown microbes in a probiotic-based drink evaluated for *Bifidobacterium* strain C that had been grown in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%).

Figure 5 shows the survival/recovery of starch-grown microbes in a yogurt type fermented milk drink evaluated for *Bifidobacterium* strain C that had been grown in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%).

Figure 6 shows the survival/recovery of starch-grown microbes in orange juice evaluated for *Bifidobacterium* strain C that had been grown in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%).

Figure 7 shows the survival/recovery of starch-grown microbes in probiotic-based drink with the inclusion of additional resistant starch evaluated for *Bifidobacterium* strain C that had been grown in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%).

Figure 8 shows the survival/recovery of starch-grown microbes in yogurt type fermented milk product with the inclusion of additional resistant starch evaluated for *Bifidobacterium* strain C that had been grown in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%).

Figure 9 shows the survival/recovery of starch-grown microbes in orange juice with the inclusion of additional resistant starch evaluated for *Bifidobacterium* strain C that had been grown in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%).

Figure 10 shows the survival/recovery of the strains exposed to a freeze-thaw cycle after growth in the presence of resistant starch. *Bifidobacterium* strain C that had been grown in the presence of glucose (0.5%), or Starch 1 (0.5%), or Starch 1 (0.25%) + glucose (0.25%).

Figure 11 shows the survival/recovery of the strains exposed to a freeze-thaw cycle after growth in the presence of resistant starch. *Bifidobacterium* strain C that had been grown in the presence of Starch 1 (0.25%) + glucose (0.25%) with addition of further Starch 1.

Figure 12 shows the survival/recovery of the strains exposed to a freeze-thaw cycle after growth in the presence of resistant starch. *Bifidobacterium* strain C that had been grown in the presence of Starch 2 (0.5%) with addition of further Starch 1.

Figure 13 shows the survival of *Bifidobacterium* strain C grown in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%) and then placed in various types of yogurts without added starch.

Figure 14 shows survival of *Bifidobacterium* strain C grown in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%) and placed in various types of yogurts with added starch.

Modes for Carrying Out the Invention

The present inventors have found that the inclusion of resistant starch to microbial growth media and optionally in subsequent stages of production of microbial preparations containing the resistant starch-grown microbes resulted in a surprising and unexpected increase in growth recovery and/or survival of the microbes during production and storage of the preparations and products.

EXAMPLE 1

Bifidobacterium strain Lafti™ 13B was anaerobically grown on a basal agar medium (BM) supplemented with 1% (w/w) of either glucose or resistant starch (Culture Pro™). After growth, cells were harvested from the plates using phosphate buffered saline (PBS) and aliquots were mixed with either PBS or PBS containing the starch granules (10% w/w). Aliquots of the mixtures were freeze dried. The susceptibility of the Lafti™ 13B cells to low pH was evaluated by adding the bacterial mixtures before freeze drying and rehydrated after freeze drying, to glycine buffer at a pH of 3.5. The viable cells were enumerated by determining colony forming units using Tryptone Yeast Extract Peptone plates (TYP) when added to pH 3.5 and after 3 hours. The reduction of viability over the 3 hours is presented in Table 1. It was noted that cells grown in the presence of starch were more resistant and that the inclusion of the starch further enhanced the resistance.

Table 1. The reduction of viability over the 3 hours of Bifidobacterium cells

	Presence of starch after growth	Reduction of viability
Glucose grown cells	-	26×10^2
Glucose grown cells	+	15×10^2
Starch grown cells	-	26
Starch grown cells	+	5

EXAMPLE 2

Bifidobacterium strain Lafti™ 13B was pre-cultured in Basal broth (BM) supplemented with 1% w/v glucose or high amylose maize starch granules (Culture Pro™). Anaerobically grown cultures were inoculated (10 µl) onto BM agar or into broth, both media containing 1% (w/v) glucose or Culture Pro™. Plates were either spot inoculated or spread and then incubated anaerobically for 48 hr. Growth in broth or cells harvested from spread plates were quantified by enumerating the colony forming units (CFU). Growth on spot-inoculated plates was quantified by measuring the size of the colony as well as the size of the cleared zone around the colony which was indicative of utilisation of the starch by the Bifidobacterium cells. It was noted that Lafti™ 13B grew more rapidly on starch-containing medium when pre-cultured using starch-containing medium and produced larger colonies and cleared zones on agar plates containing starch. Furthermore, the yield was greater from starch-containing media, compared to glucose media, for cells pre-cultured in both control broth (glucose) and starch broth.

RESULTS

Recovery of viable microorganisms after growth in the presence of starch was higher and more rapid than glucose controls.

Growth in starch media enhanced the yield of microorganisms after exposure to stress conditions, for example, low pH, bile acids, acids, heat, moisture, pressure, freeze drying, spray drying, singly or in combination.

Pre-culture in starch medium prior to growth in starch medium enhanced recovery/survival after exposure to stress conditions as outlined above, as well as enhancing yield.

Growth in starch medium and then addition of starch enhanced resistance to stress conditions as outlined above.

The invention is further illustrated by the following examples using two starches containing resistant starch, referred to as Starch 1 (about 20% (w/w) resistant starch) and Starch 2 (about 60% (w/w) resistant starch) which were granular high amylose maize starches, as well as a number of *Bifidobacterium* strains, referred to as strains A, B, C, D, E and F.

EXAMPLE 3

The growth and yield of *Bifidobacterium* strain D, *Bifidobacterium* strain E and *Bifidobacterium* strain C in the presence of natural granular maize starch was studied. A pre-culture of the strain was grown in 20 ml PYG broth for 18 hours and used to inoculate (0.1 ml aliquots) 20 ml of PY growth medium containing either glucose (0.5%), Starch 1 (0.5%), Starch 2 (0.5%), or a mixture of Starch 1 (0.25%) + glucose (0.25%). The cultures were incubated in an anaerobic chamber at 37^{0}C and sampled at 0, 4, 7, 12, 24, 31, 48 and 72 hours to monitor the number of viable cells determined as colony forming units per ml of culture (CFU/ml^{-1}). The results presented in Figure 1 for *Bifidobacterium* strain D show that the inclusion of Starch 1 (0.25%) together with 0.25% glucose resulted in enhanced growth and yield of strain D compared to 0.5% glucose alone. For *Bifidobacterium* strain E (Figure 2) growth was more rapid in Starch 2 broth compared to the glucose control. Growth for strain E was also enhanced in Starch 1 + glucose broth compared to the glucose control. A different growth pattern and yield for *Bifidobacterium* strain C was noted. As shown in Figure 3, while the growth rate was not markedly different for the various broths, this strain when grown in glucose, died off rather rapidly once the maximum yield was obtained at about 18 hours. The strain maintained a higher yield when Starch 1 or Starch 2 were included.

EXAMPLE 4

The survival/recovery of starch-grown microbes in foods was evaluated for *Bifidobacterium* strain C that had been grown in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%). The bifidobacterium cells were collected after 40 hours of anaerobic growth and diluted in the food product to yield approximately 10^5 CFU per ml of food. The foods trialed included orange juice, a probiotic based drink and a yoghurt type fermented milk product. The food products containing the *Bifidobacterium* strain C cells were stored at room temperature (for accelerated storage conditions) and sampled at 0, 1, 2, 5 and 6 days to quantify the survival of *Bifidobacterium* strain C cells. From the results presented in Figure 4, enhanced survival of strain C in the probiotic based drink occurred when the strain was previously cultured in the presence of Starch 1 or 2. Similarly, enhanced survival of strain C was

noted in the yoghurt type fermented milk product. For starch grown cells (Figure 5) and in orange juice for starch grown cells (Figure 6).

EXAMPLE 5

The inclusion of additional resistant starch to microorganisms cultured in the presence of resistant starch resulted in enhanced survival when the microorganisms were added to food products. This was investigated using *Bifidobacterium* strain C that had been grown in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%). The bifidobacterium cells were collected after 40 hours of anaerobic growth and mixed with 2.5% Starch 1 for 1 hour. The cells plus starch granules were then collected by centrifuging and added to the food product to yield approximately 10^5 CFU per ml of food. The foods trialed included orange juice, a probiotic based drink and a yoghurt type fermented milk product. The food products containing the *Bifidobacterium* strain C cells with additional starch were stored at room temperature (for accelerated storage conditions) and sampled at 0, 1, 2, 5 and 6 days to quantify the survival of *Bifidobacterium* strain C cells. As presented in Figure 7, the inclusion of additional Starch 1 extended survival of the bacteria in a probiotic based drink irrespective what growth medium was used, and, as seen in Figure 8, in yoghurt type fermented milk product. The additional starch also extended survival of strain C in orange juice (Figure 9).

EXAMPLE 6

Using *Bifidobacterium* strain C, 20 g female mice were orogastrically intubated with a suspension of cells harvested from glucose based plates or Starch 1 or Starch 2 based plates. The bacterial suspensions were standardised to a consistent optical density corresponding to approximately 10^8 per ml of diluent. The bifidobacterium cells in freshly void faecal samples were enumerated using PAM agar plates and identification confirmed using PCR. Faecal samples were collected at 4, 10, 24 and 48 hours after administration of the bifidobacterium. No bifidobacterium cells were detected in mice dosed with glucose grown cells (Table 2). Mice dosed with strain C grown on Starch 1 agar had approximately log 7 CFU per gram wet weight in faeces detected 4 and 10 hours after oral administration. Numbers decreased in the 24 and 48 hours samples but were still detectable. When *Bifidobacterium* strain C grown on Starch 2 agar was orogastrically dosed to mice, approximately log 8 CFU per gram wet weight of faeces were

detected 4 and 10 hours after administration, with a decrease for the 24 and 48 hour samples. It was concluded that growth on resistant starch based agar results in bifidobacterium cells which were more resistant to *in vivo* conditions which would include low stomach pH , bile acids and pancreatic enzymes.

Table 2. Recovery of *Bifidobacterium* strain C from freshly void faeces of mice orogastrically dosed with *Bifidobacterium* strain C cells which had been cultured on either glucose agar, Starch 1 agar or Starch 2 agar. Results expressed as CFU per gram wet weight of faeces.

Time after dosage (h)	Glucose agar	Starch 1 agar	Starch 2 agar
	(CFU per g wet weight)		
4	ND	5.8×10^6	2.5×10^8
10	ND	2.5×10^7	6.0×10^7
24	ND	$> 10^6$	$> 10^6$
48	ND	$> 10^5$	$> 10^5$

ND - none detected

EXAMPLE 7

Microorganisms cultured in the presence of resistant starch were shown to be more resistant to physical stress as illustrated by the following example in which bifidobacterium strains were exposed to a freeze-thaw cycle after growth in the presence of resistant starch. *Bifidobacterium* strain C that had been grown in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%). The bifidobacterium cells were collected after 40 hours of anaerobic growth and the samples ere divided into two. One portion was frozen directly (-20^0C) and the other portion was mixed with 2.5% Starch 1 for 1 hour. The cells plus starch granules were then collected by centrifuging and also frozen at -20^0 C. The viable bifidobacterium cells were quantified after 0, 1, 2, 3, and 4 freeze thaw cycles which involved thawing the samples at room temperature each day for 4 days. As presented in Figure 10, growth in the presence of resistant starch prior to exposure to the physical stress enhanced survival of the bifidobacterium. Furthermore, the addition of more resistant

starch before freezing improved further the survival of the Bifidobacterium cells (Figures 11 and 12).

EXAMPLE 8

Bacteria grown in the presence of resistant starch and subsequently freeze dried prior to storage at elevated temperatures were more resistant to elevated temperatures than those grown in the absence of the starch. Three *Bifidobacterium* strains A, B and D were each grown in fermentors with pH control. The cell biomass was harvested and freeze dried. The dried powder was then stored at 42^{0}C for 7 days and sampled daily for 4 days and then again on day 7 to quantify the viable bifidobacteria. The results are expressed as the colony forming units per gram of dried powder. As illustrated in Table 3, cells that were grown in the presence of Starch 1, survived better or were recovered at higher levels than those grown in the absence of the resistant starch.

Table 3. Survival of freeze dried *Bifidobacterium* strains A, B and D at 42^{0}C incubation

	Strain A		Strain B		Strain C	
Time (d)	No starch	With starch	No starch	With starch	No starch	With starch
0	10.52	10.59	10.58	10.56	10.65	10.58
1	10.30	10.23	10.23	10.40	10.11	10.20
2	9.11	10.11	7.83	10.08	7.76	9.83
3	7.26	10.00	5.00	9.53	5.00	8.53
4	4.60	9.48	4.00	8.08	4.00	5.68
7	0.00	8.04	0.00	0.00	0.00	1.30

EXAMPLE 9

The survival of *Clostridium butyricum* was monitored after growth in broth with no added starch. The cells were harvested and resuspended in pH 3.8 buffer containing 2.5% Starch 1 , 2.5% Starch 2, 2.5% cellulose or 2.5% starch 3 and the resultant viable cells quantified after 3 hours. The results are expressed as the percentage relative to the initial viable count. As can be seen in Table 4, the presence of Starch 2 enhanced survival. The cellulose did not protect against loss of viability as was noted for the resistant starch.

Table 4. Survival of *Clostridium butyricum* grown in the absence of resistant starch and then combined with 2.5% Starch 1, 2 or 3 suspended in buffer of pH 3.8. The loss of viability was monitored after 3 hours exposure to the pH 3.8.

Starch addition	Percentage viable after 3 hours at pH 3.8
No starch	80.5
Starch 1	71.3
Starch 2	111.3
Cellulose	ND
Starch 3	66.0

ND = none detected

EXAMPLE 10

It was shown that there is synergy between food ingredients such as polysaccharides and the enhanced effect on survival of microorganisms when grown in the presence of resistant starch and/or additional resistant starch is included with the microbes. This can be demonstrated by the growth of *Bifidobacterium* strain C in the presence of glucose (0.5%), or Starch 1 (0.25%) + glucose (0.25%), or Starch 2 (0.5%), or Starch 2 (0.25%) + glucose (0.25%). The bifidobacterium cells were collected after 40 hours of anaerobic growth and the samples ere divided into two. One portion was used directly and the other portion was mixed with 2.5% Starch 1 for 1 hour. The cells alone or the cells plus starch granules were then collected by centrifuging and resuspended in a yoghurt type fermented milks which were then stored at room temperature and sampled at 0, 1, 2, 5, and 6 days for viable bifidobacterium testing. As can be seen in Figures 13 and 14, there was a

demonstrable synergy between the presence of polysaccharide in yoghurt B and the growth in the presence of resistant starch compared to glucose.

EXAMPLE 11

It was established that bacterial cells attached to a surface survive stress conditions better than unattached cells. In this example it was found that cells of bifidobacterium grown in resistant Starch 1 adhered two-fold better to Starch 1 granules than was noted for glucose grown cells. Cells of *Bifidobacterium* strain C were grown in PYG for 24 hours and after harvesting by centrifugation were resuspended in a solution of 2.5% Starch 1 at pH 7.0 or at pH 2.5. After one hour incubation, the adhesion to the starch granules was assessed and then the pH was altered from 7.0 to 2.5 and also from 2.5 to 7.0. As can be seen in Table 5, cells of strain C adhered well at pH 7.0 but not at pH 2.5 and when the pH was changed from 7.0 to 2.5, the cells remained attached while there was an increase in adhesion when the pH was raised from pH 2.5 to pH 7.0.

Table 5. The effect of pH on adhesion of *Bifidobacterium* strain C to Starch 1

Measurement at 1h		Measurement at 2h	
pH	Adhesion (%)	pH	Adhesion (%)
7.0	88.8	7.0	86.7
2.5	18.3	2.5	13.4
7.0	88.1	pH change to 2.5	75.5
2.5	19.51	pH changes to 6.8	72.8

USES

(i) The invention can be applied to situations for which probiotic microbes may be used, including use as prophylactic and therapeutic agents as well as in food and feed compositions for the benefit of the host.

(ii) The invention can be applied to situations for which probiotic microbes may be used for non-digestive tract applications like the nasal and vaginal tracts.

(iii) The invention can be applied to situations relating to biocontrol and bioremediation.

(iv) The probiotic microorganisms can be grown in the starch-based medium and used directly or combined with additional starch after growth.

These probiotic suspensions may be used directly or after freezing and/or drying in the absence or presence of further additives .

(v) The probiotic microorganisms described in (ii) above, can be added to foods and feeds either during or at the end of production.

(vi) In addition to the foods and feeds described in (iii) above, the starch may also be added to the food or feed prior to or after addition of the probiotic microorganisms.

(vii) The invention also applies to microorganisms, including starter cultures, used for production of fermented foods wherein these microorganisms are grown in starch-based media and optionally mixed with additional starch after growth, freezing and/or drying, thereby enhancing survival of the microbes. When these microbes are added as an ingredient, additional starch may be added to the food before, during or after production.

The invention covers the fact that for many different microorganisms, including probiotic bacteria such as lactic acid bacteria and bifidobacteria, the presence of resistant starch in the growth media can in solid and liquid preparations:

increase the growth and/or yield of the microorganism; and

increase the survival rate or recovery rate of the microbes in food, health foods including nutraceutical and/or functional foods, health supplements, food and food formulations designed for infants and geriatrics, pharmaceutical products, medical foods such as enteral feeding preparations, animal feeds, feeds for companion animals, aquaculture, bird feeds and supplements, sport and performance food supplements.

Although the examples provided related mainly to food and probiotic products, it will be appreciated that other microorganisms useful for different applications, for example biocontrol and bioremediation, may be rendered to have increased growth/yield potential, or increased survival/recovery rate in use.

Growth and survival can be enhanced if additional resistant starch is added to the microorganisms after growth, and even when added to cells grown in the absence of resistant starch and which are subsequently mixed with starch.

Furthermore, the microorganisms grown in the presence of resistant starch are more resistant to stresses such as aeration, shear, freezing, drying, freeze drying, high temperature, low temperature, temperature fluctuations,

pressure fluctuations, high pressure, low pressure, low pH, high pH, moisture, *in vivo* conditions.

The resistant starch, including RS1, RS2, RS3 and RS4 types, can be natural starches containing resistant starch and/or modifications thereof including both chemical and enzymatic modifications, and/or mixtures thereof. Examples of two different starches were used in the examples but one skilled in the art would appreciate that other forms of resistant starch would also be suitable for use in the present invention.

The above cited enhanced growth/yield and /or survival or recovery also applies to production and storage of preparations of microorganisms.

In addition, it was surprising to note a synergy with other food ingredients including disaccharides, oligosaccharides and polysaccharides when growth/yield and/or survival or recovery of microorganisms was monitored. It would be appreciated that other food ingredients such as proteins and fats may also act synergistically.

It was also noted that resistant starch grown cells adhered better to starch granules which in turn would ensure better survival or recovery. Since the resistant starch has reduced digestibility, it would be appreciated that other indigestible compounds including proteins and lipids could also provide enhanced growth/yield and/or survival or recovery of microorganisms.

The prior art has shown that the presence of resistant starch in probiotic compositions enhances survival of the probiotic microorganisms during and after consumption (AU 687253). This earlier patent by the present applicants shows data using bifidobacteria with additional starch added. In contrast, the present invention results from the unexpected discovery that resistant starch grown cells are more robust without the addition of more starch. Surprising, when the microorganisms are grown in the presence of resistant starch, and may have additional starch added to the starch grown cells, enhanced growth/yield and /or survival or recovery of the microorganism occurs. That is, microbial cells grown in the presence of resistant starch are more robust. Furthermore, the addition of resistant starch to cells grown in the absence of starch can enhance the robustness of those cells.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.